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(57) Abstract

PDGF AB–dependent regulation of endothelial cell gene expression, particularly in PDGF–α a receptor positive cardiac microvascular endothelial cells which constitutively express PDGF–A, is described, as well as methods of using the disclosed pathway to regulate endothelial cell development and function.

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PDGF-MEDIATED MICROVASCULAR COMMUNICATION AND METHODS OF USE THEREOF

RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No.60/064,951, filed November 7, 1997, the entire teachings of which are incorporated herein by reference.

GOVERNMENT SUPPORT

Work described herein was supported in part by the National Institute of Health. The United States government has rights in the subject invention.

BACKGROUND OF THE INVENTION

The diversity of cellular and tissue functions within organs requires that local communication circuits control distinct populations of cells. Endothelial cells of different microvascular beds carry out organ-specific functions, such as regulating angiogensis and modulating hemostasis, by expressing discrete sets of gene products. Endothelial cell diversity is particularly well documented in relation to the hemostatic activity of different vascular beds. For example, previous studies have established that plasminogen activator inhibitor-1 is expressed at high levels in vascular beds of the murine aorta, heart and adipose tissue, and at low levels in the vascular beds of liver, adrenals, and kidney(Sawdey and Loskutoff, *J. Clin. Invest. 88*:1346-1353 (1991). Heterogenous gene expression can even be observed within different regions of a single vascular bed, as exemplified by the observation that higher levels of thrombomodulin are expressed in abdominal regions of the murine aorta

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relative to the level expressed in the thoracic region of the same vessel(Weiter-Guettler *et al., Circ. Res. 78*:180-187 (1996). Differences in gene expression patterns are also found between microvascular and macrovascular endothelial cells (Speiser *et al., Blood 69*:964-967 (1987).

These differences in local hemostatic regulation represent only a fraction of known endothelial cell heterogeneity, which is known to also extend to the growth characteristics and angiogenic activity of endothelial cells. For example, endothelial cells derived from arterial, venous and microcirculatory beds are characterized by noticeably different basal mitotic rates, and endothelial cells from macrovascular versus microvascular beds exhibit different growth responses to the addition of exogenous growth factors, including the expression of PDGF isoforms and receptors (Shimada *et al., Artery, 18*:268-284 (1991; D'Amore *et al., Growth Factors 8*: 61-75 (1993). Taken together, these observations suggest that the regulation of endothelial cell function is the basis of diversity in the vascular system and that organ-specific endothelial cell gene expression is controlled by an interplay between local enviornmental factors and intracellular transcriptional networks.

Understanding the interplay between local environmental factors and intracellular transcriptional networks could provide insight into intercellular cellular communication pathway which enable organs to specifically control microvascular endothelial cell gene expression.

SUMMARY OF THE INVENTION

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The present invention is based on the elucidation of a platelet derived growth factor AB(referred to herein as PDGF AB)dependent pathway of microvascular communication between cardiac myocytes and microvascular endothelial cells, which allows for the extravascular regulation of endothelial cell gene expression *in vivo*, *ex vivo* and *in vitro*. More specifically, the present invention describes a cardiac microvascular communication pathway that is capable of regulating the expression of endothelial cell genes involved in hemostatis and angiogenesis.

The invention pertains to regulators and methods of regulating the development and function of microvascular endothelial cells comprising regulating the interaction of PDGF AB with the high affinity PDGF α receptor(referred to herein as PDGF-Rα)expressed on the surface of endothelial cells. Specifically encompassed by the present invention are substances, or regulators, that regulate the interaction of PDGF AB with endothelial cell PDGF-Rα. More specifically, the invention relates to a method wherein the regulated endothelial cell function (also referred to herein as activity) is selected from the group of activities consisting of proliferation, chemotactic migration, angiogenesis, neovascularization and hemostatic activities. In a particular embodiment of the invention, the angiogenic activity of microvascular endothelial cells is regulated by inducing the expression of the endothelial cell mitogen vascular endothelial growth factor (referred to herein as VEGF) and its receptor Flk-1. In another embodiment of the present invention, the hemostatic activity of microvascular endothelial cells is regulated by inducing the expression of von Willebrand factor (referred to herein as vWF).

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The invention also relates to a method of regulating tissue specific microvascular endothelial cell gene expression in PDGF-Rα positive endothelial cells that constitutively express PDGF A comprising contacting the endothelial cells with a soluble factor, produced by cells residing in the local microenvironment, which induces endothelial cell expression of PDGF B, thereby resulting in the formation of PDGF AB heterodimers. For example, the factor can be derived from cardiac myoctytes, and the factor's ability to mediate the induction of endothelial cell gene expression can be characterized by being susceptible to neutralization by interaction with an anti- epidermal growth factor (referred to herein as EGF) antibody. In an alternative embodiment the soluble factor comprises exogenous PDGF AB. In a particular embodiment, the regulated gene is selected from the group consisting of vWF, VEGF, Flk-1 and other genes characterized by encoding a promoter region that is responsive to PDGF AB-mediated PDGF-Rα signal transduction.

The invention also pertains to a method of promoting angiogenesis by PDGF-Rα positive microvascular endothelial cells which constitutively express the PDGF A polypeptide chain, comprising contacting endothelial cells with a soluble factor which induces microvascular endothelial cell expression of PDGF B polypeptide chain, thereby resulting in the formation of the AB isoform of PDGF, which upon interaction with its high affinity (e.g. PDGF-Rα) receptor induces microvascular endothelial cell expression of VEGF and Flk-1.

The invention further pertains to a method of inhibiting PDGF AB-mediated PDGF-Rα dependent signal transduction in microvascular endothelial cells comprising contacting endothelial cells with a regulator substance (also referred to as an agent), that blocks

PDGF AB binding to its corresponding receptor. In one embodiment, the agent is an antibody, polyclonal or monoclonal, antibody fragment or a single chain antibody, which is characterized by an ability to bind to an eptiope that is present in the A or B polypeptide chain of PDGF, or in the α or β polypeptide subunit of PDGF receptor chains, or an epitope created by the formation of the dimeric PDGF ligand. In an alternative embodiment, the substance is a rationally designed small molecule, or an oligopeptide, or a recombinant PDGF polypeptide chain or dimer which competes with, and thereby antagonizes, the binding of the endogenously produced PDGF to its corresponding receptor expressed on the surface of endothelial cells.

The invention also relates to a method of inhibiting PDGF AB-mediated VEGF/Flk-1 dependent neovascularization in microvascular endothelial cells comprising contacting PDGF-Rα positive microvascular endothelial cells expressing PDGF AB heterodimers with a substance, regulator or agent, that inhibits PDGF receptor dimerization, thereby preventing downstream signal transduction events (e.g. transcriptional activation) required for the initiation of neovascularization. In a particular embodiment the agent is selected from the group consisting of rationally designed small molecules, oligopeptides derived from the sequence of the PDGF A and B polypeptide chains, recombinant polypeptide chains or PDGF heterodimers, and antibodies or functional portions of antibodies.

The invention further relates to a method for evaluating a candidate substance for its ability to regulate the interaction of PDGF AB with PDGF α receptors expressed on microvascular endothelial cells comprising coculturing microvascular endothelial cells

and cardiac myocytes in transwell cultures, introducing a candidate substance that is being assayed for its ability to regulate the interaction of PDGF AB with PDGF-Rα into the coculture, and determining the relative level of endothelial cell activity in cocultures maintained in the presence and absence of the candidate substance.

Thus, based on the discovery described herein, new regulators and methods are now available for regulating the interaction of PDGF AB with PDGF-Rα, thereby providing regulations for, and methods of, modulating angiogenesis, neovascularization and hemostatis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of cardiac microvascular communication. PDGF B expression by cardiac microvascular endothelial cells is induced in the presence of cardiac myocytes. PDGF B dimerizes with PDGF A and in turn induces PDGFRα receptor positive endothelial cells to express endogenous vWF, the vWF*LacZ*-2 transgene, VEGF and Flk- 1.

Figures 2A is a graphic representation of ELISA data summarizing the relative percentages of PDGF A polypeptide chain expressed by endothelial cells cultured either alone (control) or in the presence of cardiac myocytes, or samples of recombinant PDGF AA, AB or BB isoforms.

Figures 2B is a graphic representation of ELISA data summarizing the relative percentages of PDGF B polypeptide chain expressed by endothelial cells cultured either alone (control)or in the presence of cardiac myocytes, or samples of recombinant PDGF AA, AB or BB isoforms.

Figures 3A - 3D are a set of 4 graphic representations of the protein levels of endogenous

vWF, vWF*lacZ*-2 transgene product, VEGF and Flk-1 observed in samples obtained from cardiac microvascular endothelial cells cultured either alone(control), or in the presence of either cardiac myocytes, PDGF AB, or cardiac myocytes and a neutralizing antibody to PDGF AB.

Figures 4A - 4C show the *in vivo* neutralization of PDGF AB induced expression of vWF*lacZ*-2 transgene. The photographs in Figures 4A and 4B illustrate X-Gal staining observed in 12.5 day vWF*lacZ*-2 embryo hearts injected with either an isotype control antibody(4A)or anti-PDGF antibody (4B). Figure 4C is a graphic representation of the percentage of hearts in each of the antibody treatment groups exhibiting X-Gal staining.

DETAILED DESCRIPTION OF THE INVENTION

The description herein of a cardiac microvascular communication pathway which is capable of regulating genes involved in angiogenesis and hemostasis represents the first molecularly characterized local pathway by which an organ specifically controls gene expression in endothelial cells. The disclosed model system provides a paradigm for similar tissue specific pathways that regulate the function of endothelial cells residing in alternative vascular beds in response to signals derived from their local microenvironment.

A hallmark of a local communication scheme is the regional control of gene expression in distinct populations of cells. The endothelium exhibits a remarkable diversity of cellular properties that are uniquely adapted to serve the needs of the underlying tissue, making the vascular system an excellent model in which to elucidate mechanisms of cellular diversity. Heterogeneity within the endothelium has been described at the level of cell structure, antigen composition, mRNA expression, and cell function (Aird *et al. J. Cell.*

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Biol. 138: 1117-1124(1997). The organ-specific functions of endothelial cells located in different vascular beds require a high level of phenotypic diversity, and subpopulations of microvascular endothelial cells residing in different vascular beds carry out organ-specific tasks by synthesizing discrete sets of gene products. The concept underlying the experimental approach which led to the elucidation of the described communication pathway is a model of gene regulation which predicts that the expression pattern of endothelial cell genes observed within the vascular tree reflect the combined effects of an interplay between multiple signaling pathways, that vary from one microenvironment to another, and intracellular transcription mechanisms. The envisioned array of local communication pathways provides an effective means of establishing functionally distinct endothelial cell populations which are capable of tailoring their pattern of gene expression to manifest alternative phenotypes in response to the specific needs of the surrounding tissue. The presence of a subpopulation of cells characterized by a unique transcriptional control mechanism which renders them responsive to local signals (e.g. resulting from the interaction of microvascular endothelial cells with extracellular matrix components, neighboring cells, or to the expression of paracrine and autocrine growth factors) may constitute a critical population of cells which allow the microvascular endothelium to fulfill its specific functional requirements. Such a population is herein exemplified, by the colocalization data of Example 2, which demonstrates that the expression of the transgene and PDGF-Rα were both restricted to a subpopulation of microvascular endothelial cells which constitutively express PDGF A.

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Previous studies have demonstrated a significant variation in the responsiveness of endothelial cells isolated from different vascular beds to growth factors (Shimada, *et al., Artery 18*: 268-284 (1991); D'Amore *et al., Growth Factors 8*: 61-75 (1993), including the expression of PDGF isoforms and receptors (Linder, V., *Pathobiology 63*: 257-264 (1995). This heterogeneity in responsiveness suggests that the disclosed description of a PDGF AB-mediated PDGF α receptor-depedent pathway of cardiac microvascular communication may be relatively unique to the cardiac microvasculature. However, given the tendency of nature to utilize signaling functions modularly in numerous distinct pathways, it is likely that the signaling paradigm exemplified in this system will provide insight into other local regulatory systems. Alternative systems will likely utilize distinct growth factors and their corresponding receptors to mediate similar communication pathways which will potentially confer the capability of regulating microvascular endothelial cell expression in other vascular beds.

The present invention is based on the discovery of a PDGF-mediated communication pathway which allows cardiac myoctyes to regulate gene expression in cardiac, and other, microvascular endothelial cells. Specifically, cardiac myocytes induce the expression of PDGF B polypeptide chains in a subpopulation of PDGF-Rα positive microvascular endothelial cells which constitutively produce PDGF A polypeptide chains. The resulting PDGF AB isoform binds to endothelial cell PDGF receptors resulting in the upregulation in expression of the protein products encoded by the vWF, VEGF and Flk-1 genes.

The platelet derived growth factor(PDGF)family includes three disulfide-bonded dimeric ligands,

constituted from two related polypeptide chains, PDGF A and PDGF B, which are encoded by different genes (Leveen *et al., Genes & Devel. 8*:1875-1887 (1994); Seifert, et *al., J. Biol. Chem. 268*:4473-4480 (1993). PDGF expression in the majority of cell types generally accompanies functional or mitogenic activation. All three possible isoforms, PDGF AA, PDGF BB and PDGF AB, have been isolated from natural sources, and different cell types produce characteristic mixtures of the three different dimers (Seifert, et *al., J. Biol. Chem. 268*:4473-4480 (1993)).

The biological effects of PDGF are mediated by interaction (also referred to herein as binding) with two distinct cell surface receptors: an α-receptor (PDGF-Rα) and a β-receptor(PDGF-Rβ). According to the current model of PDGF ligand-receptor interaction, PDGF-Rα binds each of the three PDGF isoforms with high affinity, whereas PDGF-Rβ binds only PDGF-BB with high affinity and PDGF-AB with lower affinity (Krupinski, *et al., Stroke 28*:564-573). The pattern of binding of PDGF isoforms to different cell types is determined by the differential ability of each of the growth factor dimers to bind to their corresponding receptor subunits. PDGF receptor dimerization occurs as a result of ligand binding and appears to be a prerequisite for signal transduction across the plasma membrane (Heldin, *et al., J. Biol. Chem. 264*:8905-8912 (1989).

As used herein, the term "PDGF" encompasses all of the above-described isoforms, as well as biologically active fragments of PDGF. Fragments of PDGF can be obtained by methods well known to those of skill in the art, including digestion and synthesis of PDGF peptides. The PDGF fragments useful in this invention are those which exhibit biological activity comparable to the

activity of intact or full length growth factor. Biologically active PDGF, and PDGF fragments, are characterized by an ability to interact with, or bind to, a PDGF receptor expressed on endothelial cells. This interaction of a biologically active PDGF fragment with a PDGF receptor will result in one, or more, of the following endothelial cell activities: dimerization of the PDGF receptor; induction of PDGF-Rα signal transduction; expression of the vWF gene and/or its protein product; expression of the VEGF gene and/or its protein product; expression of the Flk-1 gene and/or its gene product; chemotactic cellular migration; or cellular differentiation. Detection of these activities which reflect the cellular consequences resulting from a PDGF/PDGF-Rα interaction (also referred to herein as binding), can be accomplished as described in the examples provided herein, or by the use of other methods well known to one of skill in the art.

Further encompassed by the present invention are biologically active analogs, derivatives, and mutants of PDGF polypeptide chains, or PDGF receptor protein subunits. Biological activity of these PDGF/PDGF receptor analogs, derivatives and mutants can be determined as describe above.

Previous studies have established that the regulation of endothelial cell gene expression varies between blood vessel type and location of the vascular bed (Aird, *et al, J.Cell. Biol. 138*:1117-1124 (1997). For example, the multimeric glycoprotein von Willebrand factor, which is a cofactor for platelet adhesion and a carrier for the antihemophiliac factor (Ruggeri and Ware, *FASEB J.* 7:308-316 (1993), is heterogenously distributed throughout the vascular tree and is associated with variations in mRNA levels (Aird, *et al*, (1997). vWF is expressed at higher levels on the venous

side of the circulation compared with the levels observed to be expressed in arteries and and arterioles. By contrast, consistently low levels of vWF are present within the sinusoidal endothelial cells of the liver and spleen. Histochemical analysis reveals that the The vWF protein is present in clusters of endothelial cells oriented along the longitudinal axis of blood flow. The *in vivo* administration of thrombin results in an increase in histochemically detectable vWF expression, suggesting that cells which do not constitutively express vWF, can be induced to do so (Senis et al.,Br. J. Hematol. 93: 195-203 (1996). These data suggest that the transcriptional control of vWF varies from one endothelial cell to another and that cell-to-cell variation may be programmed by signals from the local microenvironment.

Consistent with this theory, the vWF*lacZ*-2 transgenic mice described herein, have been previously used to demonstrate that endothelial cell expression of vWF transgene under the control of a human promoter sequence is regulated *in vivo* by a distinct organ-specific transcriptional pathway, which is responsive to signals from the microenviornment. This conclusion was based on the fact that the transgene was observed to have a limited *in vivo* distribution pattern, relative to the endogenous gene, with the transgene promoter directing a limited pattern of endothelial cell subtype restricted expression in transgenic mice.

It was further observed that in *in vitro* coculture assays, similar to the cultures described herein, and in the vascular beds of auricular tissue which received isogenic embryonic heart grafts, the expression of both the endogenous vWF gene and the transgene in cardiac microvascular endothelial cells was upregulated in the presence of cardiac myoctyes. This data is consistent

with the theory that the critical information required for cardiac microvascular specific transcriptional activation of the transgene is not contained within the endothelial cell, but rather is determined by signals originating in the microenvironment. vWF is a key factor in the regulation of hemostasis, thus regulating the interaction of PDGF AB with the PDGF-Rα results in the regulation of vWF, and also hemostasis.

Previous studies have also established the importance of PDGF to the physiologic development of the cardiac vasculature. Deletion or neutralization of either PDGF A (Schatteman, *et al., Dev. Biol. 176*:133-142 (1996), PDGF B (Leveen, *et al., Genes Dev. 8*:1875-1887 (1994); Lindahl, *et al., Science 277*:242-245 (1997) , or the PDGFRα (Schatteman, *et al., Development 115*:123-131 (1992); Stephenson *et al., Proc.. Natl. Acad. Sci.* USA *88*:6-10 (1991); Morrison-Graham *et al., Development 115*:133-142 (1992), but not the PDGFRβ (Soriano, *Genes Dev. 8*:1888-1896 (1994), results in embryonic lethality with marked cardiac vascular abnormalities. In addition, mice carrying a null mutation in the gene encoding the PDGF B polypeptide chain (which therefore lack PDGF BB and PDGF AB isoforms) exhibit a phenotype which includes abnormal kidney glomeruli, heart and blood vessel dilation, anemia, thrombocytopenia and hemorrhages which invariably result in perinatal death ((Leveen, et al., *Genes Dev. 8:* 1875-1887 (1994). Overall the data from the loss-of-function studies conducted in knockout mice demonstrate that normal development of the cardiac vasculature requires intact PDGF signaling components.

Previous work has further demonstrated that the expression PDGF-Rα is regulated during angiogenesis (Marx *et al., J. Clin. Invest. 93*:131-139

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(1994), suggesting that a PDGF/PDGF α receptor communication pathway may be involved in the regulation of genes which are critical to angiogenesis. In addition, recent studies have demonstrated increases in PDGF isoforms in response to, or as a potential component of, cardiac vascular pathophysiologic conditions which induce a neovascular response, including myocardial ischemia (Ogawa *et al., Am. J. Cardiol. 69*:453-456 (1992); Ogawa *et al., Coron. Artery Dis. 4*:437-442 (1993) and allograft rejection (Shaddy, *et al., Am. J. Cardiol.* 77:1210-1215 (1996). In an adult, the putative functions of PDGF generally relate to different responses to pathological conditions. For example, PDGF has been functionally implicated in connective tissue overgrowth resulting from chronic inflammatory processes, atherosclerosis, fibrosis, and tumor stroma formation (Leveen, *et al., Genes and Devel. 8*:1875-1887 (1994). The established roles of PDGF A and B and the PDGF-Rα in cardiac development and disease further suggests that the PDGF pathway may be involved in governing the development and function of microvasculature endothelial cells in general, and the cardiac microvasculature in particular.

The observed increase in downstream elements of PDGF AB-PDGFRα signaling described herein, particularly increases in vascular endothelial growth factor (VEGF) and its receptor Flk-1, implicate a potential role for a PDGF mediated communication angiogenesis. Under the influence of specific cytokines, or angiogenic growth factors, microvascular endothelial cells change from a quiescent state to a rapidly replicating phenotype which ultimately gives rise to formation of new microvessels. Angiogenesis may be physiologic, as exemplified by endometrial cycling, or pathological, as

exemplified by the neovascularization of tumors. The endothelial cell-specific mitogen vascular endothelial cell growth factor (VEGF) is a major mediator of pathological angiogenesis, and the expression of its two receptors, Flt-1 and Flk-1 has been shown to be related to the normal development of the vasculature during embryogenesis (Ferrara *et al.*, *Nature 380*:439-442 (1996). Mice expressing homozygous mutations for either VEGF receptor die *in utero*.

Recent loss-of-function studies conducted with knockout mice have further confirmed that VEGF and Flk-1 are critical for both normal cardiac vessel development and angiogenesis. Therefore, the observed ability of PDGF mediated signaling to induce VEGF and Flk-1 expression establishes a link of this cardiac microvascular communication pathway to angiogenesis. Mice having heterologous deletions of the VEGF gene die *in utero* due to a failure of normal cardiac vessel development (Ferrara, *et al.*, *Nature 380*:439-442 (1996); Carmeliet, *et al.*, *Nature 380*:435-439 (1996). Mice possessing a single copy of the VEGF gene also exhibit early embryonic lethality, at the same stage of development at which death occurs in the corresponding null animal, providing a unique example of a situation in which a haploid gene dosage is sufficient to cause lethality (Luisa and Dvorak, *Thrombosis and Haemostasis 78*:672-677 (1997). Homozygous deletions of Flk-1 die at approximately the same time in their development as the VEGF knockout embryos and exhibit similar defects in cardiac angiogenesis (Shalaby, *et al.*, *Nature 376*:62-66 (1995). These studies establish that cardiac angiogenesis requires both VEGF and Flk-1 and suggest that their expression may be critically controlled in adult microvasculature. The instant disclosure reveals

that a PDGF AB-α receptor signaling pathway regulates the cardiac microvascular expression of VEGF and Flk-1, and that VEGF expression results in signaling through Flk-1, thereby providing a method of modulating cardiac microvascular angiogenesis.

It is widely recognized that microvasculature endothelial cells play an essential role in regulating hemostasis and angiogenesis. The described PDGF dependent pathway of cardiac microvascular communication integrates cardiac myocyte produced PDGF-mediated signals into a model of cardiac microvascular hemostatic and angiogenic regulation. Having identified the pathway, the present invention can be used to identify agents, substances, or regulators capable of regulating (or modulating) angiogenesis and hemostasis in both *in vitro* and *in vivo* situations and to identify factors which can induce the expression of microvascular endothelial cell genes. Alternatively, the invention can be used to identify agents which can inhibit the cellular effects of a PDGF/PDGF-Rα interaction either by inhibiting the binding of PDGF AB to its receptor, or alternatively by preventing the downstream signaling events associated with PDGFRα mediated signal transduction. The regulators identified by the methods described herein can be formulated into pharmaceutical compositions, or medicaments for uses in therapies to regulate the interaction of PDGF AB with the PDGF-Rα.

More specifically, the present invention also encompasses substances (also referred to herein as regulators, factors or agents) that modulate(or regulate) the cellular effects of PDGF/PDGF-Rα interactions. The type of substance useful as an inhibitor of PDGF AB-Rα receptor signal transduction is any molecule which prevents PDGF receptor dimerization, or any molecule which inhibits ligand-induced

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conformational changes in the extracellular domains of the receptor subunits that are necessary to promote receptor-receptor interactions which result in signal transduction. The type of substance useful in the present invention as an enhancer is any molecule which enhances the level of endothelial cell activity that results from a PDGF AB-Rα interaction. For example, the regulatory substance can be a rationally designed small molecule, an oligopeptide derived from the nucleotide sequence of either one of the PDGF polypeptide chains or PDGF receptor protein subunits, recombinant PDGF chain mutants or an antibody. Molecules useful in the present invention include naturally occuring or recombinant proteins, small organic molecules, synthetic peptides, biologically active fragements, antibodies, or functional portions of antibodies. For example, an oligopeptide that mimics the region of PDGF AB that interacts with PDGF-Rα, and which binds to the receptor but does not induce receptor dimerization would exemplify a useful substance. Alternatively, a recombinant PDGF polypeptide chain comprising a mutant amino acid sequence which interacts with a PDGF receptor but fails to induce a conformational change in the receptor subunit which is required for signal transduction, would be a useful subtance in the present invention. In an alternative embodiment, a recombinant molecule comprised of multimeric binding sites which efficiently crosslinks PDGF receptors would be a useful substance for inducing or enhancing (also referred to herein as modulating) the cellular effects of a PDGF AB-Rα interaction.

As used herein the term "antibody" includes both polyclonal and monoclonal antibodies or functional portions therof (e.g. an antigen binding portion such as an Fv, Fab, Fab', or F(ab)2 fragment) which binds to an

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eptiope present in the group consisting of the PGDF A polypeptide chain , PDGF B polypeptide chain , PDGFRα subunit, PDGFRβ subunit, or an epitope created by the formation of the one of the dimeric isoforms of PDGF. The type of antibodies that could act as modulators of PDGF AB binding can also be chimeric antibodies or humanized antibodies or a recombinant fusion protein.

As used herein, "inhibit" is intended to encompass any qualitative or quantitative reduction in a measured effect or charactertistic, including complete prevention, relative to a control. The term "induce" or "enhance" is intended to encompass a qualitative or quantitative increase in the level of expression of a gene, or its encoded protein product, relative to the expression level present in a control sample. The terms "modulate" and "regulate" are intended to indicate an ability to selectively control a particular activity, it is intended to encompass both the ability to inhibit or to enhance(or induce) the level of a particular cellular activity as described herein.

The invention also pertains to an assay for identifying substances which can regulate endothelial cell activity by regulating the interaction of PDGF AB with PDGF α receptors expressed on microvascular endothelial cells. The assay comprises coculturing microvascular endothelial cells and cardiac myocytes, which have been demonstrated herein to produce a soluble factor which induces the expression of PDGF B in a subpopulation of PDGF α receptor positive cardiac microvascular endothelial cells, in transwell cultures in the presence and absences of a candidate substance whose regulatory ability is being determined under conditions suitable for promoting a PDGF AB α receptor interaction. The regulatory activity of the substance is determined by comparing the level of endothelial cell

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activity observed in the presence of the substance to the level of activity observed in the absence of the candidate substance. An increase in the level of endothelial cell activity relative to the control indicates that the canditate substance promotes PDGF AB-mediated PDGF α receptor signal transduction; a decrease in the level or extent of endothelial cell activity relative to the corresponding control level indicates that the candidate substances is an inhibitor of PDGF AB-mediated PDGF α receptor signal transduction.

In addition to growth, the known cellular effects of PDGFs include chemotaxis, differentiation, and regulation of other cell functions, such as contraction or the production of extracellular matrix components, or as shown herein, the regulation of endothelial cell gene expression. Appropriate endothelial cell activities which could provide the basis of a model assay in which to identify substances that are capable of modulating endothelial cell gene expression therefore include proliferation, chemotactic migration, expression of PDGF isoforms, and gene expression. These activities can be monitored according to the protocols used in the examples provided herein or by the used of standard methods that are well known to those skilled in the art.

PDGF is produced by numerous cell types in addition to platelets, including monocytes/macrophages, smooth muscle cells, fibroblasts, placenta cytotrophoblasts, neurons, certain glial cells and endothelial cells. Any of these cell types could be utilized in endothelial transwell cocultures as potential sources of a soluble factor or regulator capable of mediating intracellular communication.

As disclosed herein, the induction of PDGF B poly-peptide chain expression is critical to the described pathway of cardiac microvascular communication.

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Previous studies of the PDGF B promoter suggest that the molecular mechanism by which cardiac myocytes mediate the induction of PDGF B expression could be by Egr-1 (a zinc finger transcriptional factor) signaling (Khachigian, *et al.*, *Science 271*:1427-1431 (1996). Therefore, factors which signal through increases in Erg-1 are prime candidates as mediators of microvascular communication. One such group of factors is the epidermal growth factor family (EGF). The EGF family is a large highly redundant group of growth factors, which has been shown to be involved in multiple biological processes, including mediating aspects of vascular physiology and pathophysiology. In general, soluble factors useful in the present invention as potential mediators of intercellular communication include growth factors and cytokines which are known to be produced by cells residing in close proximity to microvascular beds of major organs, which have corresponding receptors that are characteristically expressed on the surface of endothelial cells and which are capable of mediating signal transduction.

Also encompassed by the present invention are methods of therapy based on the modulation (e.g. inhibiting or enhancing) the cellular effects of PDGF AB/PDGF-Rα interaction. For example, the method of enhancing the cellular consequences of a of PDGF/PDGF-Rα interaction can be an *in vivo* method of mediating intracellular communication in a mammal, comprising administering to the mammal an effective amount of an agent which induces or enhances PDGF AB-mediated PDGF-Rα signal transduction. It may be clinically beneficial to modulate gene expression, or the functional consequences thereof, in selected populations of microvascular endothelial cells *in vivo*, in conditions where angiogenesis is beneficial such as promoting the

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neovascularization of a tissue (e.g. skin), or organ graft (e.g. heart), or the healing of a wound. According to the method, a therapeutically effective amount of one or more substances (e.g. a preparation comprising an inhibitor or promoter of PDGF/PDFG-Rα interactions) can be administered to an individual by an appropriate route, either alone or in combination with another drug in a pharmaceutical composition.

A variety of routes of administration are possible, including but not limited to, topical, parental (e.g. intravenous, intraarterial, intramuscular, subcutaneous injection) and inhalation (e.g. intrabronchial, intranasal, or oral inhalation) routes of administration, depending on the identity of the organ and vascular bed being targeted.
Other methods of passive or active transport of small molecules, or local delivery methods known to those of skill in the art can also be employed.

Formulation of an agent to be administered will vary according to the route of administration selected (e.g. solution, emulsion, capsule, aerosol). An appropriate composition comprising the agent to be administered can be prepared in a physiologically acceptable vehicle or carrier. For solutions or emulsion, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parental vehicles can include sodium chloride, Ringer's dextrose,, dextrose and sodium chloride, lactated Ringer's or fixed oils, for instance. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replinishers and the like (See, generally, Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Co., PA, 1985). For inhalation, the agent can be solubilized and loaded

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into a suitable dispenser for administration (e.g., an atomizer, neublizer or pressurized aerosol dispenser).

It will be appreciated that the actual preferred effective amounts of substance in a specific case will vary according to the specific substance being administered, the particular composition of the formulation, the route of administration, the particular situs of treatment, and the organism being treated. If administered to an individual, dosages for a given recipient will be determined on the basis of the individual characteristics, such as body weight, age, and the type and severity of the condition being treated.

Thus based on the regulators and methods described herein, microvascular endothelial cell functions, particularly angiogenesis and hemostasis, can be regulated for the therapeutic benefit of a mammal.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

EXAMPLES

METHODS AND MATERIALS USED IN EXAMPLES

GENERATION AND ANALYSIS OF TRANSGENIC MICE

A 5' fragment of the human von Willebrand factor (vWF) gene containing 2,182 base pairs of 5' flanking sequence, the first exon, the first intron, and the translational start site was cloned from a human genomic library (Stratagene, La Jolla, CA). Through sequential cloning steps, the human sequence was coupled to the SDK sequence, *LacZ* cDNA reporter gene and simian virus

polyadenylation signal of pSDK*lacZ*pA (gift from J. Rossant, Mount Sinai Hospital, Toronto, Canada). The resulting construct (vWF*lacZ*) was used to generate founder lines of *LacZ-2* transgenic mice. The generation and identification of transgenic mice as well as the analysis of tissue sections and whole mounts for *lacZ* activity and vWF immunohistochemistry was carried out as described in Aird, *et al., Proc. Natl. Acad. Sci.* USA *92*: 4567-4571 (1995).

CELL CULTURE

Cardiac microvascular endothelial cells (CMEC) and cardiac myocytes were isolated, cultured and characterized from both vWF*lacZ*-2 transgenic and wild type FVB mice, according to the methods of Lodge,*et al, Transplant. Proc.* 24:2816-2817 (1992) and Iwaki, *et al., J. Biol. Chem.* 265:13809-13817 (1990). Briefly, 2-5 day old mice (n=5 to 10) were sacrificed and their hearts were explanted. The tissues were mechanically dissociated, digested with collagenase-II, 2mg/mL (Worthington Biochemicals), and CMEC were then isolated on a gradient of 25-40% Percoll (Pharmacia). CMEC were cultured in DMEM containing 5% fetal calf serum, 100 µg/ml heparin, 10 µg/ml endothelial growth supplement, 1% BME vitamins, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, and 1% endothelial cell growth factor (Sigma). The CMEC were grown on flasks coated with 1% gelatin in PBS and passaged up to 5 times prior to use in a coculture assay. The cardiac myocytes were prepared according to the method of Iwaki, K, *et al.* (1990). Briefly, ventricular myocytes were prepared from the ventricles of 17.5 day old fetal mice by fragmenting the tissue with a straight-edge razor, followed by digestion with 0.5 mg/ml collagenase II (Worthington Biochemical

Corp., Freehold, NJ) and 1.0 mg/ml pancreatin (Sigma Chemical Co., St. Louis, MO) in ADS buffer (116 mM NaCl, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.4) at 37°C for 10 minutes. The cells were then harvested by centrifugation at 700g at 4°C for 5 minutes.

The endothelial cells were cultured at a density of 5 x 10^4 cell/mm^2 in 12 mm 0.4 μm pore transwell plates (Costar, Cambridge, MA) either alone or in the presence of the cardiac myocytes. Cocultures were then established by introducing cardiac myocytes into established endothelial cell cultures at a ratio of 1:1. The cells were cultured for 72 hr, with a media change at 48 hr. The cultures were then washed twice with PBS, and the media was changed to DME supplemented with 1% BSA and 20mM glucose. The cocultures were incubated for an additional 12 hrs, at which point samples of the culture media was collected for ELISA determination of the secreted PDGF isoforms.

Alternatively, some cardiac microvascular endothelial cells were cultured in the presence of recombinant human PDGF AA, AB or BB (10 ng/mL; R&D Systems, Minneapolis, MN). In addition, some of the transwell cocultures (cardiac microvascular endothelial cells and myocytes) were grown in the presence of rabbit anti-PDGF AB neutralizing antibody (20 μg/mL; R&D Systems).

TRANSFECTANTS EXPRESSING DOMINANT NEGATIVE RECEPTORS

Transfectants expressing either a dominant negative PDGF-alpha receptor, which preferentially inhibits signaling through the PDGF-Rα pathway(Mercola, *et al.*, *Genes Dev.* *4*:2333-2341 (1990), or a control with green fluorescent protein were generated using amphotropic retroviruses as described in Hawley, *et al.*, *Gene Ther.*

1: 136-138 (1994). Briefly, retrovirus was produced in the PHEONIX packaging cell line, which was plated at 10^6cells/well (in a 6 well cluster plate), and allowed to adhere overnight. The cells were transfected with the respective MSCV-neo2.1 plasmids (10 ug/plate), by calcium-phosphate coprecipitation and incubated at 37°C for 5 hr. Replication-defective retroviruses were harvested 5 hr later, and flash frozen in liquid nitrogen before use. Cardiac microvascular endothelial cells were plated into 6 well cluster plates at a density of 10^5 cells/well. After growing overnight, DEAE-Dextran (25 ug/ml) and the replication-defective retroviruses were added to the endothelial cells and were incubated at 32°C for 24 hr. Fresh media were exchanged after 24 hr, and the cells were cultured for another 48 hr at 37°C to allow for gene expression before their use in the coculture experiments described above.

TRANSCRIPT ANALYSIS

Total RNA was isolated from cultures of cardiac microvascular endothelial cells cultured for four days either alone, or in the presence of cardiac myocytes. Cellular lysates were prepared according to the method described in Aird, W.C. *et al, J. Cell. Biol. 138*: 1117-1124 (1997). In addition, total cellular RNA was also isolated from cardiac microvascular endothelial cells that were cultured either alone or in the presence of PDGF AB (10 ng/mL) for 6, 24 and 96 hr. Samples were assayed for expression of PDGF A and PDGF B message. Similarly, total RNA samples prepared from cardiac myocyte cocultures and samples from the PDGF time course experiment(samples obtained over intervals ranging from 6 to 96 hr) were assayed for the expression of VWF, *LacZ*, VEGF, Flk-1, G3PDH, and β-actin. Reverse

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transcription was performed on equal amount of RNA samples followed by polymerase chain reaction (RT-PCR) of the CDNA. The following oligonucleotides were employed as primers:

mouse vWF

(forward): 5'TGTCCAAGGTCTGAAGAAGA3'; (SEQ ID NO: 1)

(reverse): 5'CAGGACAAACACCACATCCA3'; (SEQ ID NO: 2)

mouse Flk-1

(forward): 5'CAGCTTGCTCCTTCCTCATC3'; (SEQ ID NO: 3)

(reverse): 5'TCTCCAGAGCAAACCAACCA3'; (SEQ ID NO: 4)

mouse VEGF

(forward): 5'GGATCCATGAACTTTCTGCTGCTGTCTTGG3'; (SEQ ID NO: 5)

(reverse): 5'TTCTGGCTTTGTCCTGTCTTTCTTTGG3'; (SEQ ID NO: 6)

LacZ

(forward): 5'GCATCGAGCTGGGTAATAAGCGTTGGCAAT3'; (SEQ ID NO: 7)

(reverse): 5'GACACCAGACCAACTGGTAATGGTAGCGAC3';(SEQ ID NO: 8)

mouse β-actin

(forward): 5'GTGGGCCGCTCTAGGCACCAA 3'; (SEQ ID NO: 9)

(reverse): 5'CTCTTTGATGTCACGCACGATTTC3'; (SEQ ID NO: 10)

G3PDH

(forward): 5'TGAAGGTCGGTGTGAACGGATTTGGC3';(SEQ ID NO: 11)

(reverse): 5'CATGTAGGCCATGAGGTCCACCAC3'; (SEQ ID NO: 12)

PROTEIN MEASUREMENT

Cellular and secreted protein samples were isolated from cardiac microvascular endothelial cells cultured alone, or in the presence of cardiac myocytes, or in presence of cardiac myocytes and neutralizing PDGF

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antibody, or alternatively in the presence of recombinant PDGF AA, AB or BB.

The relative level of each immunoreactive PDGF polypeptide chain was determined by two-antibody sandwich enzyme-linked immunoassay (ELISA). The capture antibody was a goat polyclonal antibody to PDGF A (AF-221-NA, R&D Systems), coated onto Immunon 2 ELISA strips (Dynatech Laboratories, Chantilly, VA). Samples (50 ul/well) of culture supernatant harvested from the cardiac endothelial cell cocultures described above were applied to the anti-PDGF coated strips and incubated for 2hrs at room temperature. The assay strips were washed 10 times with PBS, prior to the addition of rabbit polyclonal detection antibodies specific for the PDGF A and B polypeptide chains (anti-PDGF A, sc-128, Santa Cruz, Biotechnology; anti-PDGF B, ZP-215, Genzyme Diagnostics). The chain-specific detection antibodies were used at a dilution of 1:1000. The antigenic levels were determined by adding a peroxidase-labeled donkey anti-rabbit polyclonal (at a 1:1000 dilution), followed by the addition of 1,2 phenyl-enediamine substrate (0.67 g/l), and determination of the absorbance at 490 nm.

Relative antigenic levels of vWF, VEGF, and Flk-1 were determined by Western dot blotting with the following respective antibodies 082 (Dako, 1:500 dilution), sc-152 (Santa Cruz, 1:1000 dilution), and sc-315 (Santa Cruz, 1:500 dilution). A peroxidase-labeled donkey polyclonal antibody to rabbit IgG (Jackson ImmunoResearch Laboratories) was used as the secondary antibody at a dilution of 1:1000. The signal was developed with a chemiluminescence system (ECL; Amersham Corp., Arlington, IL).

Relative β-galactatosidase activities were determined by cleavage of ONPG (Sigma). All protein

analysis was performed in triplicate sets with a minimum of two independent experiments.

IMMUNOSTAINING

Adult vWF*LacZ*-2 hearts were harvested and 8 μm serial cryosections were obtained. Alternate sections were processed for detection of either the transgene or the PDGF-Rα. Transgene expression (vWF*LacZ*-2) was determined by X-Gal staining. Immunostaining for PDGF-Rα was performed with the primary and secondary antibodies sc-338 (Santa Cruz) and Cy3-conjugated anti-rabbit IgG (Jackson Immuno Research) respectively. Photomicrographs of adjacent serial sections with X-Gal and immunostaining, respectively, were superimposed. Cardiac microvascular endothelial cells and myocytes were cultured and fixed as previously described. The samples were immunostained for WF or Flk-1, with above antibodies and costained with a goat antibody to PDGFRα (R&D Systems, AF-307-NA), and secondary antibody staining with FITC-and Cy3-conjugated anti-rabbit IgG (Jackson ImmunoResearch). Duel label photomicrographs were superimposed.

IN VIVO NEUTRALIZATION OF PDGF AB

The effects of *in vivo* neutralization of PDGF AB binding was determined by the embryonic injection of antibody according to the method described in Schattemann, *et al., Dev. Biol. 176*: 133-142 (1996). Briefly, female vWF*LacZ*-2 mice were mated and examined for vaginal plugs. On day 12.5 of pregnancy the mice were anesthetized with avertin (2.5% vol/vol) and the uterus was exposed by lateral longitudinal incision. Individual amniotic sacs were injected with 2 μl anti-PGDF AB (AB-20-NA, R&D Systems) or non-immune IgG

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(Jackson Inimuno Research), at a concentration 1µg/mL, using a 100 mm diameter glass pipette and the lateral abdominal wall was sutured closed. The following morning the embryonic hearts were harvested and stained with X-Gal. The hearts were scored for the presence of *LacZ* staining, and the statical significance was determined by a Student's *t* test analysis.

EXAMPLE 1: ENDOTHELIAL CELL GENE EXPRESSION IS PROGRAMMED BY SIGNALS FROM THE TISSUE MICROENVIRONMENT

In seven independent vWF*lacZ*-2 founder lines, the X-Gal reaction product was detected within blood vessels of the brain, and in a subset of microvessels within the heart and skeletal muscle. In cardiac sections stained with X-Gal and processed for immunoperoxidase detection of endogenous vWF, the transgene and endogenous gene products colocalized in the endothelial lining of capillary vessels.

In contrast, the endothelial cells of the coronary arteries, coronary veins, penetrating arteries, and endocardium of the heart exhibited no detectable B-galactosidase activity but possessed immunoreactive endogenous vWF protein. Transgene expression was similarly absent in the vascular beds of other organs, including the liver, spleen, lung, and kidney as well as in the aorta. In RT-PCR analyses, *LacZ* mRNA was detected only in brain, heart and skeletal muscle. In contrast, mRNA from the endogenous vWF gene and from the endothelial cell restricted thrombomodulin gene was present in all tissues examined. vWF mRNA levels varied from one organ to another and correlated with transcript levels detected by ribonuclease protection assays. The observed vascular-bed specific expression pattern of vWF*lacZ*-2 suggests that expression of the vWF transgene

is regulated *in vivo* by the interaction of regional transcriptional networks with distinct promoter elements.

EXAMPLE 2: vWF*lacZ*-2 CARDIAC ENDOTHELIAL CELL BEDS INCLUDE A PDGF-ALPHA RECEPTOR POSITIVE SUBPOPULATION OF ENDOTHELIAL CELLS

Histochemical studies of adult vWF*lacZ*-2 cardiac microvascular endothelial cell beds revealed that the expression of the transgene and PDGFRα were both restricted to a subpopulation of microvascular endothelial cells. X-Gal and immunostaining of alternate serial heart sections revealed colocalization of the PDGF-Rα and the X-Gal staining in endothelial cells recovered from cocultures performed in the presence of cardiac myocytes, whereas PDGF-Rα negative cells did not stain for vWF transgene expression. This observation suggests that there is a subpopulation of endothelial cells with unique receptor(s) and/or signaling pathway(s) that regulates the expression of endogenous vWF and the transgene.

The colocalization data further suggests that the PDGF-Rα may play a regulatory role in the cardiac microvascular expression of the transgene, thereby implicating a role for PDGF in mediating transcriptional regulation of endothelial cell genes. Moreover, as the expression of the transgene recapitulates the expression of the endogenous vWF gene in the cardiac microcirculation, the expression of PDGF-Rα in these cells may identify them as a distinct subset of microvascular endothelial cells.

This colocalization of a growth factor receptor and transgene offered a potential mechanism of myocyte-mediated induction. However, while the presence of the PDGFRα on the X-Gal-positive endothelial cells

was highly suggestive of its possible involvement in transgene induction, an operable signaling pathway additionally requires the participation of a cardiac myocyte-derived or myocyte-induced ligand. The existence of this component of the putative pathway was investigated by establishing the cocultures described below.

EXAMPLE 3: CARDIAC MYOCYTE MEDIATED INDUCTION OF ENDOTHELIAL PDGF B EXPRESSION

Cardiac microvascular endothelial cells were cultured alone or in the presence of cardiac myocytes grown in transwells, to assess the potential role of PDGF involvement in the induction of cardiac microvascular gene expression. Samples of secreted protein taken from the transwell cocultures were analyzed by ELISA to determine the chain composition of the PDGF dimers. The data summarized in Figures 2A and 2B demonstrate that cardiac microvascular endothelial cells constitutively express the PDGF A isoform, leading to the formation of PDGF AA homodimers. Cardiac endothelial cells cultured in the presence of cardiac myocytes, were induced to express PDGF B, leading to the formation of PDGF heterodimers.

Both the homodimer and heterodimer isoforms of PDGF are ligands for the PDGF-Rα. The *in situ* localization data suggests that cardiac myocyte-mediated induction of PDGF-Rα positive endothelial cells gene expression likely involves the induced AB heterodimer, but not the constitutively expressed AA homodimer. This hypothesis was tested by the addition of exogenous recombinant PDGF ligands (e.g. PDGF AA, PDGF AB, or PDGF BB) to endothelial cultures. As shown in Figures 2A and 2B, addition of the PDGF AB heterodimer reproduced the cardiac myocyte-mediated induction of PGDF AB

heterodimers. In contrast, the exogenous addition of the AA isoform failed to reproduce the PDGF-mediated induction of endothelial cell gene expression, and the addition of the BB homodimer was observed to be active but with less than half of the potency of PDGF AB.

This data, together with the observed myocyte induction data, is consistent with the theory that the PDGF AB isoform plays a critical role in the regulation of cardiac microvascular endothelial cell gene expression, as exemplified by the PDGF AB-mediated induction of both endogenous vWF and transgene expression. Further evidence supporting the position that PDGF AB mediates local communication between myocytes and microvascular endothelial cells was obtained by using a PDGF-specific antibody to neutralize the ligand in culture. Neutralizing antibodies to PDGF AB were added to cocultures of the cardiac myocytes and endothelial cells. As shown in Figures 3A and 3B the presence of the antibody blocked the *in vitro* myocyte-mediated induction of both the endogenous vWF and its transgene.

EXAMPLE 4: PDGF AB HETERODIMER INDUCTION OF vWF AND vWFLacZ-2 TRANSGENE

The PDGF AB induction of vWF and vWF*LacZ*-2 was confirmed by RNA analysis. When this result is considered in light of the above reported colocalization data (indicating that vWF transgene expression is restricted to a PDGF α receptor positive subpopulation of microvascular endothelial cells) it suggests that cardiac myocyte endothelial cell communication is mediated by PDGF AB induction of PDGF-Rα signal transduction. This theory is consistent with published demonstrations of a PDGFRα requirement in high-affinity

heterodimer signal transduction (Seifert, *et al., J. Biol. Chem. 268:* 4473-4480 (1993).

The involvement of PDGF α receptor in the observed induction of microvascular endothelial cell gene expression was also confirmed by the use of transfected endothelial cells expressing dominant negative receptors, in the coculture experiments described above. The results from these experiments indicate that the inherent inhibition of PDGF-Rα signal transduction that characterizes these cells blocked the ability of cardiac myocytes to induce vWF gene expression by 95 ±5%.

EXAMPLE 5: PDGF INDUCES ENDOTHELIAL CELL GENES CRITICAL TO ANGIOGENESIS

Previous knockout studies have established that vascular endothehal growth factor (VEGF) and its receptor Flk- 1 are critical for angiogenesis and normal cardiac vessel development. Mice having heterozygous deletions of the gene for VEGF die in utero with a failure of cardiac vascular angiogenesis (Ferrara, *et al., Nature 380:* 439-442 (1996); Carmeliet, *et al., Nature 380:*435-439(1996). Homozygous deletions of Flk-1 die at approximately the same time in development as the VEGF-knockout embryos and have similar deficits in cardiac angiogenesis (Shalaby, *et al., Nature 376*:62-66 (1995). This requirement for both VEGF and Flk-1 in cardiac angiogenesis suggests that their expression may be critically controlled in the vasculature. The potential link of the regulation of both vWF and angiogenesis as described above suggests that the expression of VEGF and/or Flk- 1 may be regulated by the PDGF AB/α receptor signaling pathway.

The potential for the PDGF AB-α receptor interaction to regulate VEGF and Flk-1 in endothelial

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cells was therefore examined. Additional studies revealed that myocytes induced VEGF as well as Flk-1 gene expression. Similar to vWF and its transgene, the observed increases in VEGF and Flk-1 were comparably increased by PDGF AB. Moreover, antibody neutralization of PDGF AB also inhibited this induction. The increase of transcript in the presence of PDGF AB confirmed that, like vWF, there is inducible expression of both VEGF and its receptor (Figures 3C and 3D). VEGF and Flk-1 expression were observed to be comparably increased by PDGF AB and inhibited by antibody neutralization of PDGF AB. The increase of transcript in the presence of PDGF AB confirmed that, like vWF, there is a PDGF-mediated induction of both VEGF and its receptor. Involvement of PDGF-Rα was confirmed by immunostaining which indicated a colocalization of Flk-1 and the PDGF-Rα. The involvement of PDGF-Rα was further confirmed by the observation that the expression of the dominant negative PDGF-Rα in endothelial cells inhibited the induction of gene expression.

EXAMPLE 6: IN VIVO NEUTRALIZATION OF PDGF AB INDUCED SIGNALING

The *in situ* and *in vitro* data presented herein indicates that the PDGF AB-α receptor interaction constitutes a critical pathway in the development and function of cardiac microvessels. The *in vivo* significance of this interaction was examined by determining the effects of ligand inhibition during cardiac development. PDGF AB was blocked by the injection of a relatively small dose of neutralizing polyclonal antibody (administered at a dose which was chosen because it was known to be insufficient to effect normal cardiac development) into the amniotic fluid surrounding day 12.5 vWF*LacZ*-2 embryos. Control

embryos were injected with non-immune antibody. The following day the hearts were explanted and processed for X-Gal staining.

Figures 4A and 4B illustrate the X-Gal staining pattern representative of the majority of samples observed in each treatment group. Figure 4C summarizes the percentage of hearts that were positive for X-Gal staining following the injection of each of the antibodies (control antibody treated n=21; PDGF antibody treated, n=19). Scores of X-Gal-positive hearts demonstrated a decrease from a control value of 96% to 38% in the neutralized samples, $p < .0001$. Overall, the data suggest the presence of a similar PDGF AB/α receptor mediated communication *in vivo*.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

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CLAIMS

What is claimed is:

1. A regulator of the interaction of platelet derived growth factor AB with endothelial cell platelet derived growth factor α receptor, for use in therapy (e.g. in regulating the development and function of microvascular endothelial cells).

2. Use of a regulator of the interaction of platelet derived growth factor AB with endothelial cell platelet derived growth factor α receptor for the manufacture of a medicament for use in therapy (e.g. in regulating the development and function of microvascular endothelial cells).

3. A pharmaceutical composition comprising a regulator of the interaction of platelet derived growth factor AB with endothelial cell platelet derived growth factor α receptor.

4. A process for the manufacture of a medicament (e.g. for use in a method comprising regulating the development and function of microvascular endothelial cells) characterized in the use, as an essential constituent of said composition, of a regulator of the interaction of platelet derived growth factor AB with endothelial cell platelet derived growth factor a receptor.

5. A use according to Claim 1, wherein angiogenesis or neovascularization is regulated.

6. A use according to Claim 5, wherein angiogenesis is regulated by increased expression of vascular endothelial growth factor and Fkl-1 resulting from

interaction of platelet derived growth factor AB with endothelial cell platelet derived growth factor α receptor.

7. A use according to Claim 1, wherein hemostasis is regulated.

8. A use according to Claim 7, wherein hemostasis is regulated by increased expression of von Willebrand factor resulting from interaction of platelet derived growth factor AB with endothelial cell Platelet derived growth factor α receptor .

9. A regulator of tissue-specific gene expression in platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A, for use in therapy (e.g. in a method comprising contacting endothelial cells with exogenous platelet derived growth factor-AB).

10. Use of a regulator of tissue-specific gene expression in platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A for the manufacture of a medicament for use in therapy (e.g. in a method comprising contacting endothelial cells with exogenous platelet derived growth factor-AB).

11. A pharmaceutical composition comprising a regulator of tissue-specific gene expression in platelet derived growth factor α receptor positive microvascular endothelial calls constitutively expressing platelet derived growth factor A.

12. A process for the manufacture of a medicament (e.g. for use in a method comprising contacting endothelial cells with exogenous platelet derived growth factor-AB) characterized in the use, as an essential constituent of said composition, of a regulator of tissue-specific gene expression platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A.

13. A use according to Claim 12, wherein the regulated genes control an endothelial cell function selected from the group consisting of proliferation, chemotactic migration, angiogenesis, neovascularization, thrombosis and fibrinolysis.

14. A use according to Claim 12, wherein the regulated genes encode a promoter region adjacent to the nucleotide sequence encoding von Willebrand factor, vascular endothelial growth factor, or Flk-1 and further wherein the promoter is responsive to platelet derived growth factor AB-mediated platelet derived growth factor receptor signal transduction.

15. A use according to Claim 14, wherein the regulated gene is selected from the group consisting of von Willebrand factor, vascular endothelial growth factor, and Flk-1.

16. A soluble factor that induces endothelial cell expression of platelet derived growth factor B for use in therapy (e.g. in a platelet derived growth factor AB-dependent method of mediating extravascular regulation of gene expression in selected platelet derived growth factor α receptor

positive cardiac microvascular endothelial cells comprising contacting selected endothelial cells constitutively expressing platelet derived growth factor A with the soluble factor, thereby resulting in expression of platelet derived growth factor AB heterodimers, wherein the binding the heterodimer mediates platelet derived growth factor α receptor dimerization thereby mediating signal transduction).

17. Use of a soluble factor that induces endothelial cell expression of platelet derived growth factor B for the manufacture of a medicament for use in therapy (e.g. in a platelet derived growth factor AB-dependent method of mediating extravascular regulation of gene expression in selected platelet derived growth factor α receptor positive cardiac microvascular endothelial cells comprising contacting selected endothelial cells constitutively expressing platelet derived growth factor A with the soluble factor, thereby resulting in expression of platelet derived growth factor AB heterodimers, wherein the binding of the heterodimer mediatesplatelet derived growth factor α receptor dimerization thereby mediating signal transduction).

18. A pharmaceutical composition comprising a soluble factor that induces endothelial cell expression of Platelet derived growth factor B.

19. A process for the manufacture of a medicament (e.g. for use in a platelet derived growth factor AB-dependent method of mediating extravascular regulation of gene expression in selected platelet

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derived growth factor α receptor positive cardiac microvascular endothelial cells) comprising contacting selected endothelial cells constitutively expressing platelet derived growth factor A with a soluble factor, thereby resulting in expression of platelet derived growth factor AB heterodimers, wherein the binding of the heterodimer mediates platelet derived growth factor α receptor dimerization thereby mediating signal transduction) characterized in the use, as an essential constituent of said composition, of a soluble factor that induces endothelial cell expression of platelet derived growth factor B.

20. A use according to Claim 19, wherein the soluble factor that induces endothelial cell expression of platelet derived growth factor-B is a soluble factor produced by cardiac myocytes and further wherein the activity of the soluble factor is neutralized by anti-Epidermal growth factor antibodies.

21. A use according to Claim 19, wherein the soluble factor that induces endothelial cell expression of platelet derived growth factor B is exogneous platelet derived growth factor AB.

22. A use according to Claim 19, wherein the regulated genes are selected from the group consisting of von Willebrand factor, vascular endothelial growth factor and Flk-1.

23. A soluble factor that induces endothelial cell expression of platelet derived growth factor B for use in:

a) a platelet derived growth factor-dependent method of inducing von Willebrand factor gene expression in platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB heterodimers and subsequent binding to endothelial call platelet derived growth factor α receptors; or

b) a method of promoting angiogenesis by platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB heterodimers which bind to platelet derived growth factor α receptors on endothelial cells thereby inducing vascular endothelial growth factor and Flk-1 gene expression.

24. Use of a soluble factor that induces endothelial cell expression of platelet derived growth factor B for the manufacture of a medicament for use in:

a) a platelet derived growth factor-dependent method of inducing von Willebrand factor gene, expression in platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressingplatelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB heterodimers and

subsequent binding to endothelial cell platelet derived growth factor α receptors; or

b) a method of promoting angiogenesis by platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB heterodimers which bind to platelet derived growth factor α receptors on endothelial cells thereby inducing vascular endothelial growth factor and Flk-1 gene expression.

25. A process for the manufacture of a medicament for use in:

a) a platelet derived growth factor-dependent method of inducing von Willebrand factor gene expression in platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB heterodimers and subsequent binding to endothelial cell platelet derived growth factor α receptors; or

b) a method of promoting angiogenesis by platelet derived growth factor α receptor positive microvascular endothelial cells constitutively expressing platelet derived growth factor A comprising contacting endothelial cells with the soluble factor, thereby resulting in the formation of platelet derived growth factor AB

heterodimers which bind to platelet derived growth factor α receptors on endothelial cells thereby inducing vascular endothelial growth factor and Flk-1 gene expression,

characterized in the use, as an essential constituent of said composition of a soluble factor that induces endothelial cell expression of platelet derived growth factor B.

26. A method according to Claim 25, wherein the soluble factor that induces endothelial cell expression of platelet derived growth factor B is a soluble factor produced by cardiac myocytes and further wherein the activity of the soluble factor is neutralized by anti-epidermal growth factor antibodies.

27. A method according to Claim 25, wherein the factor that induces endothelial cell expression of platelet derived growth factor B is exogenous platelet derived growth factor AB.

28. An agent that blocks platelet derived growth factor AB binding to endothelial cell platelet derived growth factor α receptors for use in therapy (e.g. in a method of inhibiting platelet derived growth factor AB mediated platelet derived growth factor α receptor signaling in microvascular endothelial cells comprising contacting endothelial cells with the agent).

29. Use of an agent that blocks platelet derived growth factor AB binding to endothelial cell platelet derived growth factor α receptors for the manufacture of a medicament for use in therapy

(e.g. in a method of inhibiting platelet derived growth factor AB mediated platelet derived growth factor α receptor signaling in microvascular endothelial cells comprising contacting endothelial cells with the agent).

30. A pharmaceutical composition comprising an agent that blocks platelet derived growth factor AB binding to endothelial cell Platelet derived growth factor a receptors.

31. A process for the manufacture of a medicament (e.g. for use in a method of inhibiting platelet derived growth factor AB mediated platelet derived growth factor α receptor signaling in microvascular endothelial cells comprising contacting endothelial cells with an agent that blocks platelet derived growth factor AB binding to endothelial cell platelet derived growth factor a receptors) characterized in the use, an essential constituent of said composition, of said agent.

32. A medicament according to Claim 31, wherein the agent is an antibody, or functional portion of an antibody, characterized by binding to an epitope present in the group of polypeptide chains consisting of platelet derived growth factor A, platelet derived growth factor B, platelet derived growth factor α receptor and platelet derived growth factor β receptor or an epitope created by the formation of platelet derived growth factor dimeric ligands.

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33. A substance that inhibits platelet derived growth factor A-induced endothelial cell platelet derived growth factor α receptor dimerization for use in therapy (e.g. in a method of inhibiting platelet derived growth factor AB mediated Vascular endothelial growth factor/Flk-1 dependent neovascularization in cardiac microvascular endothelial cells comprising contacting platelet derived growth factor α receptor positive endothelial cells with the substance, thereby preventing downstream receptor-mediated signal transduction events required for neovascularization.

34. Use of a substance that inhibits platelet derived growth factor A-induced endothelial cell platelet derived growth factor α receptor dimerization for the manufacture of a medicament for use in therapy (e.g. in a method of inhibiting platelet derived growth factor AB mediated vascular endothelial growth factor/Flk-1 dependent neovascularization in cardiac microvascular endothelial calls comprising contacting platelet derived growth factor α receptor positive endothelial cells with the substance, thereby preventing downstream receptor-mediated signal transduction events required for neovascularization).

35. A pharmaceutical composition comprising a substance that inhibits platelet derived growth factor A-induced endothelial cell platelet derived growth factor a receptor dimerization.

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36. A process for the manufacture of a medicament (e.g, for use in a method of inhibiting platelet derived growth factor AB mediated vascular endothelial growth factor/Flk-I dependent neovascularization in cardiac microvascular endothelial cells comprising contacting platelet derived growth factor α receptor positive endothelial cells with the substance, thereby preventing downstream receptor mediated signal transduction events required for neovascularization) characterized in the use,, as an essential constituent of said composition, of a substance that inhibits platelet derived growth factor A-induced endothelial cell platelet derived growth factor α receptor dimerization.

37. A medicament according to Claim 36, wherein the agent is a competitive inhibitor of platelet derived growth factor AB selected from the group consisting of synthetic small molecules, oligopeptides, recombinant platelet derived growth factor fusion proteins and the antibodies or antibody portions of Claim 35.

38. A method of evaluating a candidate substance for its ability to regulate the interaction of platelet derived growth factor AB with platelet derived growth factor α receptors expressed on microvascular endothelial comprising the steps of:

a) coculturing microvascular endothelial cells in the presence of cardiac myocytes;

b) introducing into the coculture of step a) an amount of the candidate substance sufficient to regulate the interaction of platelet derived growth factor AB with platelet derived growth factor α receptors, and maintaining the

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culture under conditions and for time sufficient to induce platelet derived growth factor AB/platelet derived growth factor α receptor interaction;

c) determining the induction of platelet derived growth factor AB/platelet derived growth factor α receptor interaction in the culture of step b); and

d) comparing the induction of platelet derived growth factor AB/platelet derived growth factor α receptor interaction the culture of step b) with induction of endothelial cell activity in a co-culture of microvascular endothelial cells and myocytes cultured in the absence of candidate substance, wherein an increase of endothelial cell activity in the presence of the candidate substance indicates that the substance regulates platelet derived growth factor AB/platelet derived growth factor α receptor interaction.

39. The method of Claim 38, wherein the endothelial cell activity is selected from a group consisting of: cardiac angiogenesis; cardiac neovascularization; thrombosis and fibrinolysis.

40. A substance that regulates the interaction of platelet derived growth factor AB with platelet derived growth factor α receptor identified by the method of Claim 39.

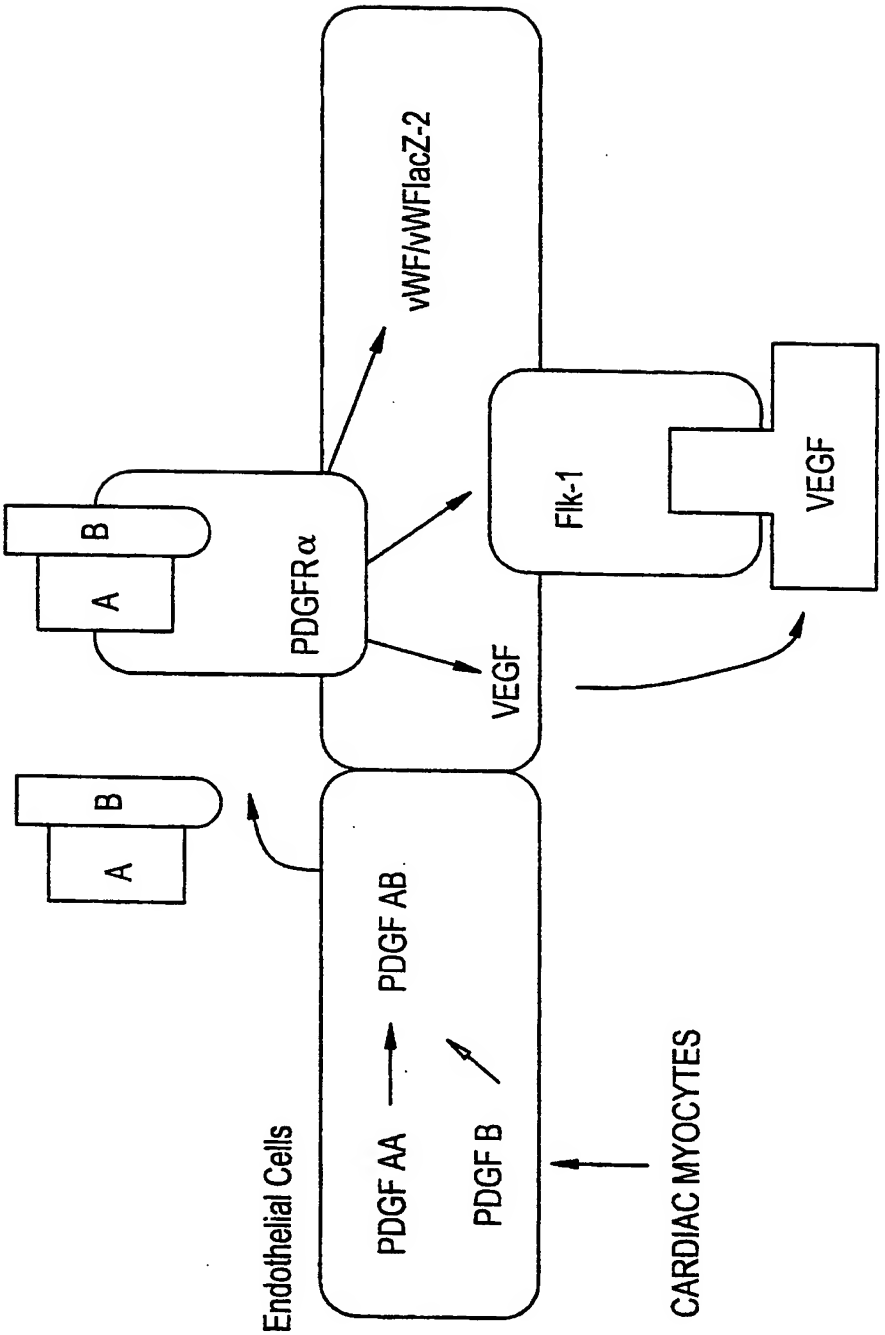

FIG. 1

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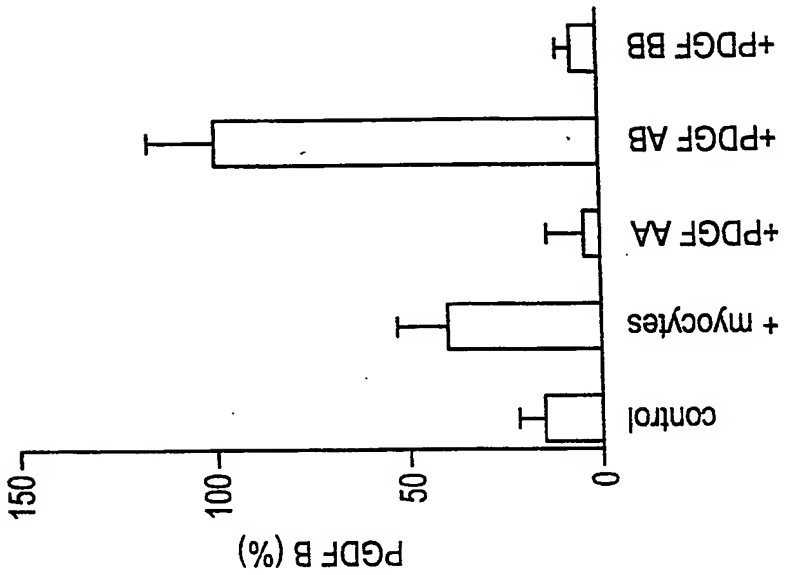

FIG. 2B

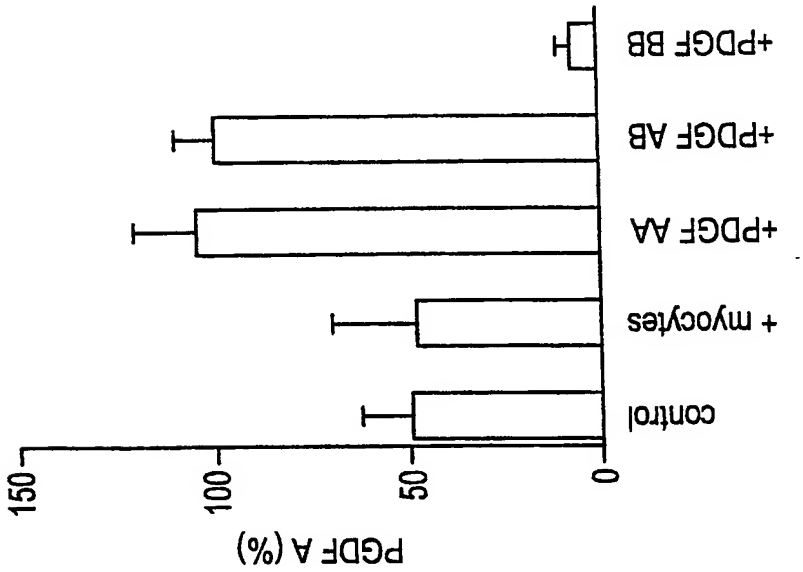

FIG. 2A

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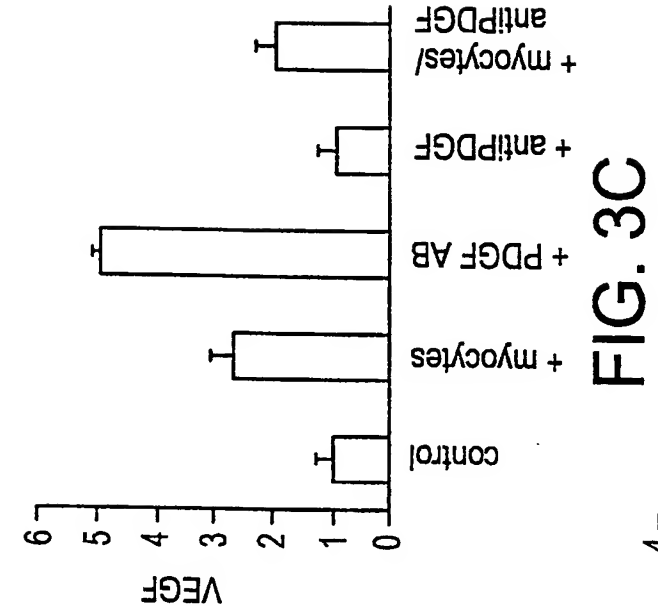

FIG. 3C

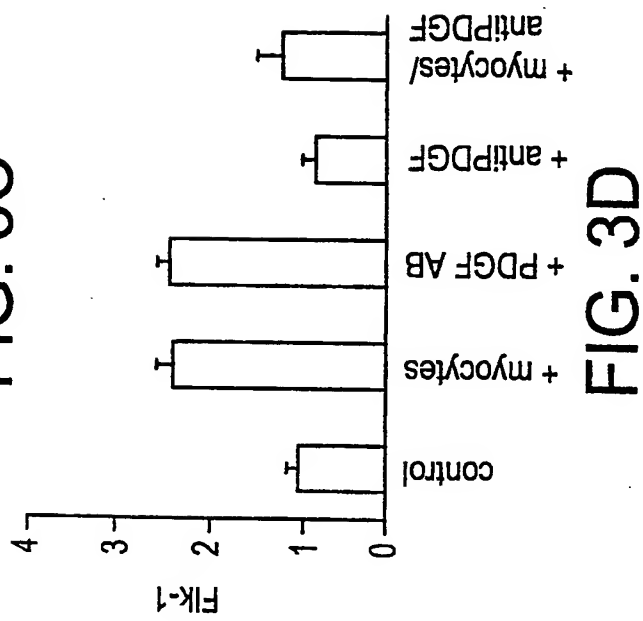

FIG. 3D

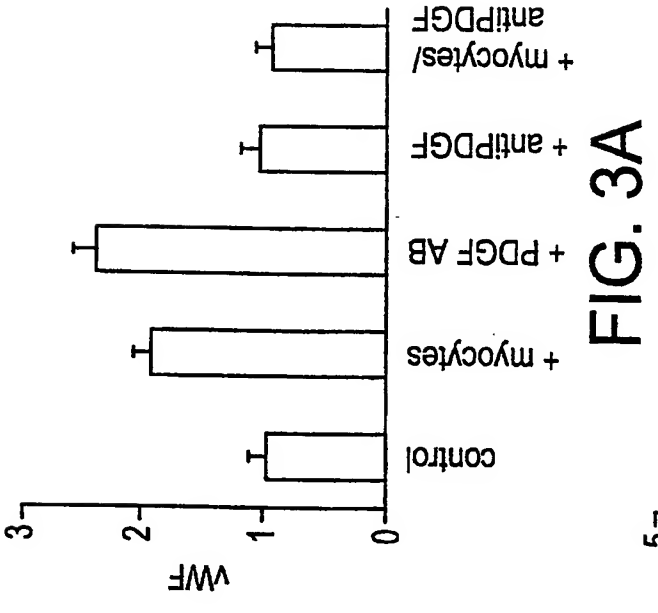

FIG. 3A

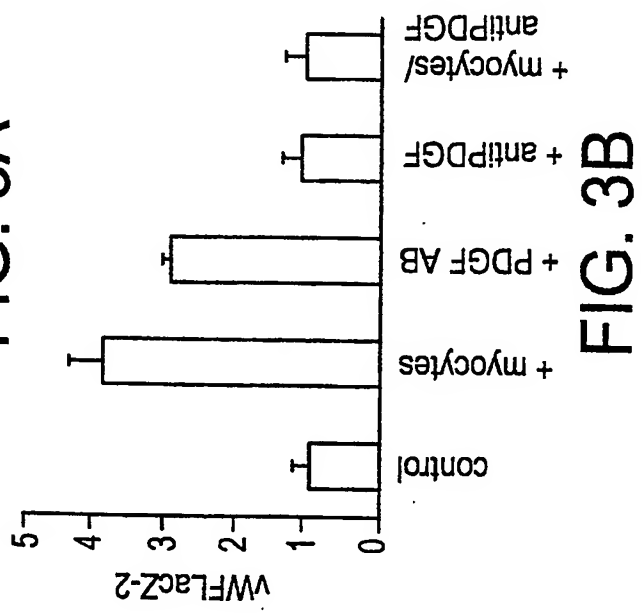

FIG. 3B

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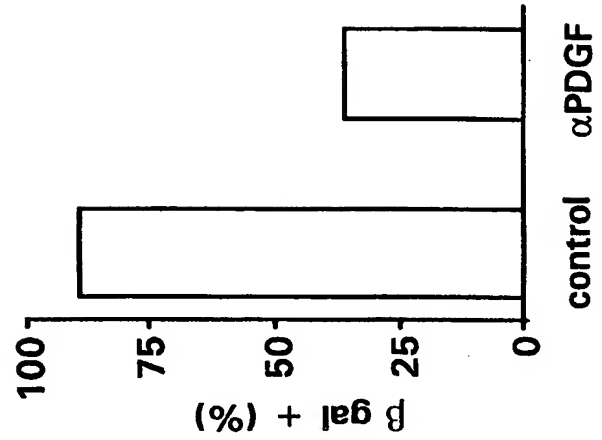

FIG.4C

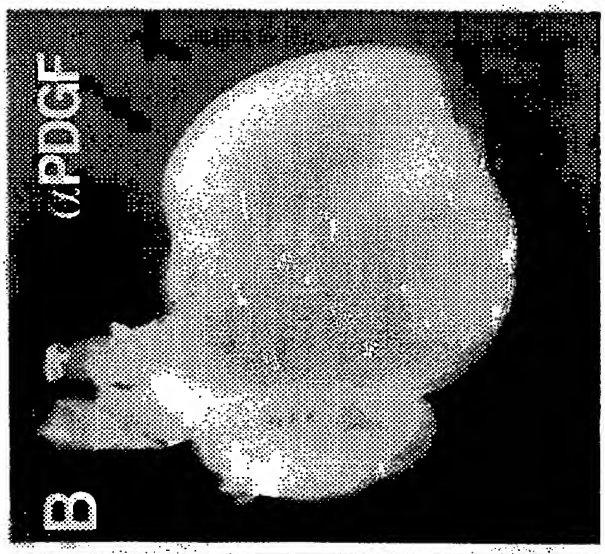

FIG.4B

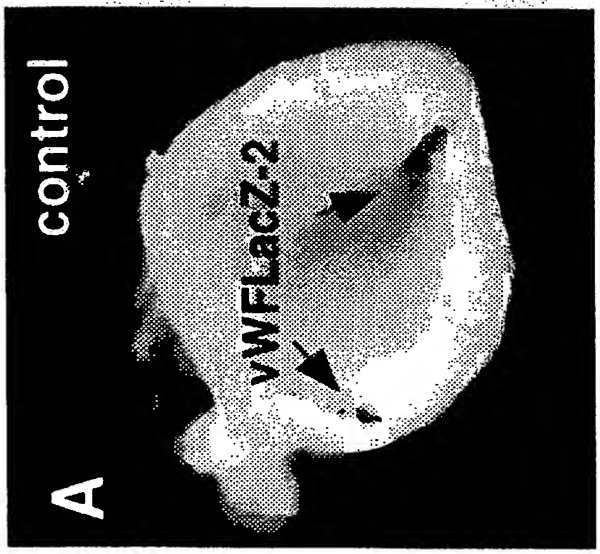

FIG.4A

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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/23892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/18 A61K39/395 A61K35/34 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.M. EDELBERG ET AL.: "CARDIAC MYOCYTE REGULATION OF CARDIAC MICROVASCULAR ENDOTHELIAL CELL GENE EXPRESSION: A CRITICAL ROLE FOR PDGF." CIRCULATION, vol. 96, no. 8 SUPPL., 21 October 1997, page I 414 XP002099041 DALLAS, TX, US see abstract nr. 2314 --- -/--	1-37

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 April 1999	16/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	W.C. AIRD ET AL.: "VASCULAR BED-SPECIFIC EXPRESSION OF AN ENDOTHELIAL CELL GENE IS PROGRAMMED BY THE TISSUE MICROENVIRONMENT." THE JOURNAL OF CELL BIOLOGY, vol. 138, no. 5, 8 September 1997, pages 1117-1124, XP002099042 NEW YORK, N.Y., US cited in the application see page 1123, right-hand column, paragraph 3 - page 1124, left-hand column, paragraph 1	1-37
A	R.A. SEIFERT ET AL.: "PDGF-AB REQUIRES PDGF RECEPTOR ALPHA-SUBUNITS FOR HIGH-AFFINITY, BUT NOT FOR LOW-AFFINITY, BINDING AND SIGNAL TRANSDUCTION." THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 6, February 1993, pages 4473-4480, XP002099043 BALTIMORE, MD, US cited in the application see page 4480, left-hand column, paragraph 2	1-37
A	WO 90 08163 A (J. HOPPE ET AL.) 26 July 1990 see claims 12,13	1-37
A	WO 94 19016 A (ZYMOGENETICS, INC. ET AL) 1 September 1994 see claims	1-37
P,X	J.M. EDELBERG ET AL.: "PDGF MEDIATES CARDIAC MICROVASCULAR COMMUNICATION." THE JOURNAL OF CLINICAL INVESTIGATION, vol. 102, no. 4, 15 August 1998, pages 837-843, XP002099044 NEW YORK, N.Y., US see the whole document	1-37

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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